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Gene coding for androctonine, vector containing it and
disease-resistant transformed plants obtained

The present invention relates to a DNA
5 sequence coding for androctonine, to a vector
containing it for the transformation of a host organism
and to the process for transforming the said organism.

The invention relates more particularly to
the transformation of plant cells and plants and to the
10 androctonine produced by the transformed plants, giving
them resistance to diseases, in particular diseases of
fungal origin.

There is today an increasing need to make
plants resistant to diseases, in particular fungal
15 diseases, in order to reduce, or even avoid altogether,
the need for treatments with antifungal protection
products, in order to protect the environment. One
means of increasing this disease-resistance consists in
transforming the plants so that they produce substances
20 capable of defending them against these diseases.

Various substances of natural origin are
known, in particular peptides, which have bactericidal
or fungicidal properties, especially against the fungi
responsible for plant diseases. However, the problem
25 consists in finding such substances which not only can
be produced by transformed plants, but also can
conserve their bactericidal or fungicidal properties
and confer these properties to the said plants. For the

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purposes of the present invention, the terms
bactericidal and fungicidal are understood to refer
both to the actual bactericidal or fungicidal
properties and to the bacteriostat or fungistat
5 properties.

Androctonines are peptides produced by
scorpions, in particular from the species *Androctonus*
australis. An androctonine and its preparation by
chemical synthesis are described by Ehret-Sabatier et
10 al., along with its *in vitro* antifungal and
antibacterial properties.

The androctonine genes have now been
identified, and it has also been found that they can be
inserted into a host organism, in particular a plant,
15 in order to express an androctonine, both for the
preparation and isolation of this androctonine and to
give the said host organism properties of resistance to
fungal diseases and to diseases of bacterial origin,
thereby providing a particularly advantageous solution
20 to the problem outlined above.

The subject of the invention is thus,
firstly, a nucleic acid fragment coding for an
androctonine, a chimeric gene comprising the said
fragment coding for an androctonine and heterologous
25 regulation elements in positions 5' and 3' which can
function in a host organism, in particular in plants,
and a vector for transforming host organisms containing
this chimeric gene, and the host organism transformed.

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The invention also relates to a transformed plant cell containing at least one nucleic acid fragment coding for an androctonine, and to a disease-resistant plant containing the said cell, in particular a plant regenerated from this cell. Lastly, the invention relates to a process for cultivating transformed plants according to the invention.

According to the invention, the term androctonine is understood to refer to any peptide which can be produced by and isolated from scorpions, in particular from the species *Androctonus australis*, these peptides comprising at least 20 amino acids, preferably at least 25, and 4 cysteine residues which form disulphide bridges between themselves.

Advantageously, the androctonine essentially comprises the peptide sequence of general formula (I) below:

Xaa-Cys-Xab-Cys-Xac-Cys-Xad-Cys-Xae

(I)

in which

Xaa represents a peptide residue comprising at least 1 amino acid,
Xab represents a peptide residue of 5 amino acids,
Xac represents a peptide residue of 5 amino acids,
Xad represents a peptide residue of 3 amino acids, and
Xae represents a peptide residue comprising at least 1 amino acid.

Advantageously, Xab and/or Xad and/or Xae comprise at least one basic amino acid, preferably 1.

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According to the invention, the term basic amino acids is understood to refer to amino acids chosen from lysine, asparagine and homoasparagine.

Preferably,

- 5 Xaa represents the peptide sequence Xaa'-Val, in which Xaa' represents NH₂ or a peptide residue comprising at least 1 amino acid, and/or
- Xab represents the peptide sequence -Arg-Xab'-Ile, in which Xab' represents a peptide residue of 3 amino
- 10 acids, and/or
- Xac represents the peptide sequence -Arg-Xac'-Gly-, in which Xac' represents a peptide residue of 3 amino acids, and/or
- Xad represents the peptide sequence -Tyr-Xad'-Lys, in
- 15 which Xad' represents a peptide residue of 1 amino acid, and/or
- Xae represents the peptide sequence -Thr-Xae', in which Xae' represents COOH or a peptide residue comprising at least 1 amino acid.

20 Preferably,

- Xaa' represents the peptide sequence Arg-Ser-, and/or
- Xab' represents the peptide sequence -Gln-Ile-Lys-, and/or
- Xac' represents the peptide sequence -Arg-Arg-Gly-,
- 25 and/or
- Xad' represents the peptide residue -Tyr-, and/or
- Xae' represents the peptide sequence -Asn-Arg-Pro-Tyr.

According to a preferred embodiment of the

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invention, androctonine is represented by the peptide sequence of 25 amino acids described by the sequence identifier No. 1 (SEQ ID NO. 1) and the homologous peptide sequences.

5 The term homologous peptide sequences is understood to refer to any equivalent sequence comprising at least 65% homology with the sequence represented by the sequence identifier No. 1, it being understood that the 4 cysteine residues and the number
10 of amino acids separating them remain identical, certain amino acids being replaced with different but equivalent amino acids on sites which do not induce a substantial change in the antifungal or antibacterial activity of the said homologous sequence. Preferably,
15 the homologous sequences comprise at least 75% homology, more preferably at least 85% homology and even more preferably 90% homology.

 The NH₂-terminal residue of androctonine can exhibit a post-translational modification, for example
20 an acetylation, while the C-terminal residue can exhibit a post-translational modification, for example an amidation.

 The expression peptide sequence essentially comprising the peptide sequence of general formula (I)
25 is understood to refer not only to the sequences defined above, but also to such sequences comprising, at one or other of their ends or at both ends, peptide residues required for their expression and targeting in

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a host organism, in particular a plant cell or plant.

This in particular concerns a "peptide-
androctonine" or "androctonine-peptide", advantageously
"peptide-androctonine", fusion peptide whose cleavage
5 by the enzymatic systems of the plant cells allows the
release of the androctonine defined above. The peptide
fused to androctonine can be a signal peptide or a
transit peptide which allows the production of
androctonine to be controlled and oriented specifically
10 in one part of the host organism, in particular of the
plant cell or plant, such as, for example, the
cytoplasm or the cell membrane, or in the case of
plants, in a specific type of cell or tissue
compartment or in the extracellular matrix.

15 According to one embodiment, the transit
peptide can be a chloroplast-addressing signal or a
mitochondrion-addressing signal, which is then cleaved
off in the chloroplasts or the mitochondria.

According to another embodiment of the
20 invention, the signal peptide can be an N-terminal
signal or "prepeptide", optionally in combination with
a signal responsible for retaining the protein in the
endoplasmic reticulum, or a vacuole-addressing peptide
or "propeptide". The endoplasmic reticulum is the site
25 at which maturation operations on the protein produced,
such as, for example, cleavage of the signal peptide,
are undertaken by the "cell machinery".

The transit peptides can be single or double,

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and, in this case, optionally separated by an
intermediate sequence, i.e. one comprising, in the
direction of transcription, a sequence coding for a
transit peptide of a plant gene which codes for a
5 plastid localization enzyme, a portion of sequence of
the N-terminal mature portion of a plant gene coding
for a plastid localization enzyme, and then a sequence
coding for a second transit peptide of a plant gene
coding for a plastid localization enzyme, as described
10 in patent application EP 0,508,909.

As transit peptide which is useful according
to the invention, mention may be made in particular of
the signal peptide of the tobacco PR-1 α gene
(WO 95/19443), represented with its coding sequence by
15 the sequence identifier No. 2 (SEQ ID NO. 2) and fused
to androctonine by the sequence identifier No. 3 (SEQ
ID NO. 3), in particular corresponding to the fusion
protein corresponding to bases 12 to 176 of this
sequence, in particular when the androctonine is
20 produced by plant cells or plants, or the precursor of
Mat α 1 factor when the androctonine is produced in
yeasts.

The present invention thus relates, firstly,
to a nucleic acid fragment, in particular a DNA
25 fragment, coding for the androctonine defined above.
According to the invention, this can be a fragment
isolated from *Androctonus australis*, or alternatively a
derived fragment, adapted for the expression of

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androctonine in the host organism in which the peptide will be expressed. The nucleic acid fragment can be obtained according to the standard methods for isolation and purification, or alternatively by synthesis according to the usual techniques of successive hybridizations of synthetic oligonucleotides. These techniques are described in particular by Ausubel et al.

According to the present invention, the expression "nucleic acid fragment" is understood to refer to a nucleotide sequence which can be of DNA or RNA type, preferably of DNA type, in particular cDNA, especially of double-stranded type.

According to one embodiment of the invention, the nucleic acid fragment coding for androctonine is the DNA sequence described by the sequence identifier No. 1 (SEQ ID NO. 1), a homologous sequence or a sequence complementary to the said sequence, more particularly the coding portion of this SEQ ID NO. 1, corresponding to bases 1 to 75.

According to the invention, the term "homologous" is understood to refer to a nucleic acid fragment having one or more sequence modifications when compared with the nucleotide sequence described by the sequence identifier No. 1 coding for androctonine. These modifications can be obtained according to the usual mutation techniques, or alternatively by selecting the synthetic oligonucleotides used in the

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preparation of the said sequence by hybridization. With regard to multiple combinations of nucleic acids which can lead to the expression of the same amino acid, the differences between the reference sequence described by the sequence identifier No. 1 and the homologous sequence can be considerable, and all the more so when it concerns a DNA fragment less than 100 nucleic acids in size, which can be produced by synthesis.

Advantageously, the degree of homology will be at least 70% relative to the reference sequence, preferably at least 80% and more preferably at least 90%. These modifications are generally neutral, i.e. they do not affect the primary sequence of the resulting androctonine.

The present invention also relates to a chimeric gene (or expression cassette) comprising a coding sequence and heterologous regulation elements in positions 5' and 3' which can function in a host organism, in particular plant cells or plants, these elements being functionally linked to the said coding sequence, the said coding sequence comprising at least one DNA fragment coding for androctonine as defined above (including the "peptide-androctonine" or "androctonine-peptide" fusion peptide).

The term host organism is understood to refer to any lower-order or higher-order monocellular or multicellular organism into which the chimeric gene according to the invention can be introduced, for the

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production of androctonine. Such organisms are, in particular, bacteria, for example *E. coli*, yeasts, in particular yeasts of the genera *Saccharomyces* or *Kluyveromyces*, *Pichia*, fungi, in particular
5 *Aspergillus*, a baculovirus, or, preferably, plant cells and plants.

According to the invention, the term "plant cell" is understood to refer to any plant-derived cell which can constitute undifferentiated tissues such as
10 calli, differentiated tissues such as embryos, plant portions, plants or seeds.

According to the invention, the term "plant" is understood to refer to any differentiated multicellular organism capable of photosynthesis, in
15 particular monocotyledons or dicotyledons, more particularly crop plants which may or may not be intended for human or animal consumption, such as corn, wheat, rapeseed, soybean, rice, sugar cane, beetroot, tobacco, cotton, etc.

20 The regulation elements required for the expression of the DNA fragment coding for androctonine are well known to those skilled in the art as a function of the host organism. They comprise in particular promoter sequences, transcription
25 activators, transit peptides and termination sequences, including start and stop codons. The means and methods for identifying and selecting the regulation elements are well known to those skilled in the art.

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For the transformation of microorganisms such as yeasts or bacteria, the regulation elements are well known to those skilled in the art and comprise, in particular, promoter sequences, transcription
5 activators, transit peptides, termination sequences and start and stop codons.

In order to direct the expression and secretion of the peptide in the yeast culture medium, a DNA fragment coding for hellomycin is incorporated into
10 a shuttle vector which comprises the following elements:

- markers which allow the transformants to be selected,
- a nucleic acid sequence which allows replication (origin of replication) of the plasmid in the yeast,
- 15 - a nucleic acid sequence which allows replication (origin of replication) of the plasmid in *E. coli*,
- an expression cassette consisting of
 - (1) a promoter regulation sequence,
 - (2) a sequence coding for a signal peptide
 - 20 (or prepeptide) combined with an addressing peptide (or propeptide),
 - (3) a polyadenylation or terminator regulation sequence.

These elements have been described in several
25 publications, including Reichhart et al., 1992, Invert. Reprod. Dev., 21, pp. 15-24 and Michaut et al., 1996, FEBS Letters, 395, pp. 6-10.

Preferably, yeasts from the species *S.*

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cerevisiae are transformed with the expression plasmid by the lithium acetate method (Ito et al., 1993, J. Bacteriol, 153, pp. 163-168).

The invention relates more particularly to
5 the transformation of plants. As promoter regulation sequence in plants, it is possible to use any promoter sequence of a gene which is naturally expressed in plants, in particular a promoter of bacterial, viral or plant origin such as, for example, that of a gene for
10 the small subunit of ribulose biscarboxylase/oxygenase (RuBisCO) or of a plant virus gene such as, for example, that of cauliflower mosaic virus (CAMV 19S or 35S), or a promoter which can be induced by pathogens such as tobacco PR-1a, it being possible to use any
15 suitable known promoter. Preferably, use is made of a promoter regulation sequence which favours the overexpression of the coding sequence in a constitutive manner or induced by the attack of a pathogen, such as, for example, that comprising at least one histone
20 promoter as described in patent application EP 0,507,698.

According to the invention, it is also possible to use, in combination with the promoter regulation sequence, other regulation sequences which
25 are located between the promoter and the coding sequence, such as transcription activators ("enhancers"), such as, for example, the tobacco mosaic virus (TMV) translation activator described in patent

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application WO 87/07644, or the tobacco etch virus
(TEV) translation activator described by Carrington &
Freed.

As polyadenylation or terminator regulation
5 sequence, it is possible to use any corresponding
sequence of bacterial origin, such as, for example, the
nos terminator of *Agrobacterium tumefaciens*, or
alternatively of plant origin, such as, for example, a
histone terminator as described in patent application
10 EP 0,633,317.

According to the present invention, the
chimeric gene can also be combined with a selection
marker adapted to the transformed host organism. Such
selection markers are well known to those skilled in
15 the art. Such a marker may be an antibiotic-resistance
gene or alternatively a herbicide-tolerance gene for
plants.

The present invention also relates to a
cloning or expression vector for the transformation of
20 a host organism containing at least one chimeric gene
as defined above. Besides the above chimeric gene, this
vector comprises at least one origin of replication
and, where appropriate, a suitable selection marker.
This vector can consist of a plasmid, a cosmid, a
25 bacteriophage or a virus, which are transformed by
introducing the chimeric gene according to the
invention. Depending on the host organism to be
transformed, such transformation vectors are well known

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to those skilled in the art and are widely described in the literature.

For the transformation of plant cells or plants, such a vector is, in particular, a virus which
5 can be used for the transformation of the plants developed and also containing its own replication and expression elements. Preferably, the vector for transforming the plant cells or plants according to the invention is a plasmid.

10 The subject of the invention is also a process for transforming host organisms, in particular plant cells, by incorporating at least one nucleic acid fragment or one chimeric gene as defined above, it
15 being possible for this transformation to be obtained by any suitable known means, which is amply described in the specialized literature, and in particular the references cited in the present application, more particularly by means of the vector according to the invention.

20 One series of methods consists in bombarding cells, protoplasts or tissues with particles to which the DNA sequences are attached. Another series of methods consists in using, as a means of transfer into the plant, a chimeric gene inserted into a Ti plasmid
25 of *Agrobacterium tumefaciens* or an Ri plasmid of *Agrobacterium rhizogenes*.

Other methods can be used, such as microinjection or electroporation, or alternatively

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direct precipitation using PEG.

A person skilled in the art will select the appropriate method as a function of the nature of the host organism, in particular the plant cell or plant.

5 The subject of the present invention is also transformed host organisms, in particular plant cells or plants, containing an effective amount of a chimeric gene comprising a sequence coding for the androctonine defined above.

10 The subject of the present invention is also plants containing transformed cells, in particular plants regenerated from the transformed cells. The regeneration is obtained by any suitable process which depends on the nature of the species, as described, for
15 example, in the above references.

For the processes for transforming plant cells and for regenerating plants, mention will be made in particular of the following patents and patent applications: US 4,459,355,

20 US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073, EP 267,159, EP 604,662, EP 672,752, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318, US
25 5,204,253, US 5,405,765, EP 442,174, EP 486,233, EP 486,234, EP 539,563, EP 674,725, WO 91/02071 and WO 95/06128.

The subject of the present invention is also

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the transformed plants obtained from the cultivating and/or crossing of the above regenerated plants, as well as the seeds of transformed plants.

The plants thus transformed are resistant to certain diseases, in particular to certain fungal or bacterial diseases. Consequently, the DNA sequence coding for androctonine can be inserted with the main aim of producing plants that are resistant to the said diseases, since androctonine is effective against fungal diseases such as those caused by *Cercospora*, in particular *Cercospora beticola*, *Cladosporium*, in particular *Cladosporium herbarum*, *Fusarium*, in particular *Fusarium culmorum* or *Fusarium graminearum*, or by *Phytophthora*, in particular *Phytophthora cinnamomi*.

The chimeric gene may also advantageously be combined with at least one selection marker, such as one or more herbicide-tolerance genes.

The DNA sequence coding for androctonine can also be inserted as a selection marker during the transformation of plants with other sequences coding for other peptides or proteins of interest, such as, for example, herbicide-tolerance genes.

Such herbicide-tolerance genes are well known to those skilled in the art and are described in particular in patent applications EP 115,673, WO 87/04181, EP 337,899, WO 96/38567 or WO 97/04103.

Needless to say, the transformed cells and

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plants according to the invention can also comprise the
sequence coding for androctonine, other heterologous
sequences coding for proteins of interest, such as
other complementary peptides capable of giving the
5 plant resistance to other diseases of bacterial or
fungal origin, and/or other sequences coding for
herbicide-tolerance proteins, in particular defined
above and/or other sequences coding for insect-
resistance proteins, such as the Bt proteins in
10 particular.

The other sequences can be inserted using the
same vector comprising the chimeric gene according to
the invention, which comprises a sequence coding for
androctonine, and comprising at least one other gene
15 comprising another sequence coding for another peptide
or protein of interest.

They can also be inserted using another
vector comprising at least the said other sequence,
according to the usual techniques defined above.

20 The plants according to the invention can
also be obtained by crossing parents, one carrying the
gene according to the invention coding for
androctonine, the other carrying a gene coding for at
least one other peptide or protein of interest.

25 Among the sequences coding for other
antifungal peptides, mention may be made of the one
coding for drosomycin, described in patent application
Fr 2,725,992 and by Fehlbauer et al., (1994), and in the

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unpublished patent application FR 97/09115 filed on 24
July 1997.

Lastly, the present invention relates to a
process for cultivating transformed plants according to
5 the invention, the process consisting in planting the
seeds of the said transformed plants in an area of a
cultivation environment, in particular a field, which
is suitable for cultivating the said plants, in
applying an agrochemical composition to the said area,
10 without substantially affecting the said transformed
seeds or plants, and then in harvesting the plants
cultivated when they reach the desired maturity, and
optionally in separating the seeds from the harvested
plants.

15 According to the invention, the term
agrochemical composition is understood to refer to any
agrochemical composition comprising at least one active
product having either herbicidal, fungicidal,
bactericidal, virucidal or insecticidal activity.

20 According to a preferred embodiment of the
cultivation process according to the invention, the
agrochemical composition comprises at least one active
product having at least a fungicidal and/or
bactericidal activity, more preferably having an
25 activity complementary to that of the androctonine
produced by the transformed plants according to the
invention.

According to the invention, the expression

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product having activity complementary to that of
androctonine is understood to refer to a product having
a complementary spectrum of activity, i.e. a product
which will be active against attacks by androctonine-
5 insensitive contaminants (fungi, bacteria or viruses),
or alternatively a product whose spectrum of activity
totally or partially covers that of androctonine, and
whose dose of application will be substantially reduced
on account of the presence of the androctonine produced
10 by the transformed plant.

Lastly, cultivation of the transformed host
organisms allows the large-scale production of
androctonine. The subject of the present invention is
thus also a process for preparing androctonine,
15 comprising the steps of cultivating the transformed
host organism comprising a gene coding for androctonine
as defined above in an appropriate cultivation
environment, followed by the extraction and total or
partial purification of the androctonine obtained.

20 The examples below make it possible to
illustrate the invention, the preparation of the
sequence coding for androctonine, the chimeric gene,
the integration vector and the transformed plants. The
attached Figures 1 to 5 describe schematic structures
25 of certain plasmids prepared for the construction of
the chimeric genes. In these figures, the various
restriction sites are marked in *italics*.

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Example 1: Construction of the chimeric gene

All the techniques used below are standard laboratory techniques. The detailed procedures for these techniques are described in particular in Ausubel et al.

PRPA-MD-P: Creation of a plasmid containing the signal peptide for the tobacco PR-1a gene.

The two complementary synthetic

- 10 oligonucleotides Oligo 1 and Oligo 2 below are hybridized at 65°C for 5 minutes and then by slowly decreasing the temperature to 30°C over 30 min.

Oligo 1: 5' GCGTCGACGC GATGGGTTTC GTGCTTTTCT CTCAGCTTCC
15 ATCTTTCCTT CTTGTGTCTA CTCTTCTTCT TTTCC 3'
Oligo 2: 5' TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA
GAAGAGTAGA CACAAGAAGG AAAGATGGAA GC 3'

- After hybridization between Oligo 1 and
20 Oligo 2, the remaining single-stranded DNA serves as a matrix for the klenow fragment of *E. coli* polymerase 1 (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the
25 3' end of each oligo. The double-stranded oligonucleotide obtained is then digested with the restriction enzymes *SacII* and *NaeI* and cloned in the plasmid pBS II SK(-) (Stratagene) digested with the

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same restriction enzymes. A clone comprising the region coding for the signal peptide of the tobacco PR-1a gene (SEQ ID NO. 2) is thus obtained.

- 5 PRPA-PS-PR1a-andro: Creation of a sequence coding for androctonine fused to the PR-1a signal peptide without an untranscribed 3' region.

The two complementary synthetic oligonucleotide sequences Oligo 3 and Oligo 4 are
10 hybridized according to the operating conditions described for pRPA-MD-P.

Oligo 3: 5' AGGTCCGTGT GCAGGCAGAT CAAGATCTGC AGGAGGAGGG
GTGG 3'

15 Oligo 4: 5' CCGGATCCGT CGACACGTTT GCCTCGCCGA GCTCAGTATG
GCCTGTTAGT GCACTTGTAG TAGCAACCAC CCCTCCTCCT
GCAGATCTTG ATCTGCC 3'

After hybridization between Oligo 3 and
20 Oligo 4, the remaining single-stranded DNA serves as a matrix for the klenow fragment of *E. coli* polymerase 1 (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the
25 3' end of each oligo. This double-stranded oligonucleotide containing the portion coding for androctonine (SEQ ID NO. 1) is then cloned directly in the plasmid pRPA-MD-P, which was digested with the

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restriction enzyme *NaeI*. The correct orientation of the clone obtained is verified by sequencing. A clone comprising the region coding for the PR-1a-androctonine fusion protein, located between the *NcoI* restriction site at the N-terminal end and the *ScaI*, *SacII* and *BamHI* restriction sites at the C-terminal end (SEQ ID NO. 3), is thus obtained.

pRPA-RD-238: Creation of an expression vector in plants comprising the sequence coding for the PR-1a androctonine fusion protein.

The plasmid pRTL-2 GUS, derived from the plasmid pUC-19, was obtained from Dr. Jim Carrington (Texas A&M University, not described). This plasmid, whose schematic structure is represented in Figure 1, contains the duplicated CaMV 35S promoter isolated from cauliflower mosaic virus (CaMV 2x35S promoter; Odell et al., 1985) which directs the expression of an RNA containing a 5' untranslated sequence of tobacco etch virus (TEV 5' UTR; Carrington and Freed, 1990), the *E. coli* β -glucuronidase gene (GUS; Jefferson et al., 1987) followed by the CaMV RNA 35S polyadenylation site (CaMV polyA; Odell et al., 1985).

The plasmid pRTL-2 GUS is digested with the restriction enzymes *NcoI* and *BamHI* and the main DNA fragment is purified. The plasmid pRPA-PS-PR1a-andro is digested with the restriction enzymes *NcoI* and *BamHI* and the small DNA fragment containing the region coding

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for the PR-1a-androctonine fusion protein is purified.
The two purified DNA fragments are then linked together
in an expression cassette in the plants which
synthesizes a PR-1a-androctonine fusion protein. The
5 schematic structure of this expression cassette is
represented in Figure 2. "PR-1a-androctonine"
represents the region coding for the PR-1a-androctonine
fusion protein of pRPA-RD-230. The androctonine is
transported to the plant's extracellular matrix by the
10 action of the PR-1a peptide signal.

**pRPA-RD-195: Creation of a plasmid containing a
modified multiple cloning site.**

The plasmid pRPA-RD-195 is a plasmid derived
15 from pUC-19 which contains a modified multiple cloning
site. The complementary synthetic oligonucleotides
Oligo 5 and Oligo 6 below are hybridized and made
double-stranded according to the procedure described
for pRPA-MD-P.

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Oligo 5: 5' AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC
GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG
CATGC 3'

Oligo 6: 5' CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT
25 GCATGCCTGC AGGTGACTC TAGAGG 3'

The double-stranded oligonucleotide obtained
is then inserted into pUC-19, which was predigested

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with the restriction enzymes *EcoRI* and *HindIII* and made blunt at the ends using the klenow fragment of *E. coli* DNA polymerase 1. A vector containing multiple cloning sites to facilitate the introduction of the expression
5 cassettes into an *Agrobacterium tumefaciens* vector plasmid is obtained. The schematic structure of this multiple cloning site is represented in Figure 3.

PRPA-RD-233: Introduction of the PR-1a-androctonine

10 **expression cassette from pRPA-RD-230 into pRPA-RD-195.**

The plasmid pRPA-RD-230 is digested with the restriction enzyme *HindIII*. The DNA fragment containing the PR-1a-androctonine expression cassette is purified. The purified fragment is then inserted into pRPA-RP-
15 195, which was predigested with the restriction enzyme *HindIII* and dephosphorylated with calf intestinal phosphatase.

PRPA-RD-174: Plasmid derived from pRPA-BL-150A (EP

20 **0,508,909) containing the bromoxynil-tolerance gene from pRPA-BL-237 (EP 0,508,909).**

The bromoxynil-tolerance gene is isolated from pRPA-BL-237 by means of a PCR gene amplification. The fragment obtained has blunt ends, and is cloned in
25 the pRPA-BL-150A *EcoRI* site, the ends of which were made blunt by the action of klenow polymerase under standard conditions. An *Agrobacterium tumefaciens* vector which contains the bromoxynil-tolerance gene

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close to its right-hand end, a kanamycin-tolerance gene close to its left-hand end and a multiple cloning site between these two genes is obtained.

The schematic structure of pRPA-RD-174 is represented in Figure 4. In this figure, "nos" represents the polyadenylation site of *Agrobacterium tumefaciens* nopaline synthase (Bevan et al., 1983), "NOS pro" represents the *Agrobacterium tumefaciens* nopaline synthase promoter (Bevan et al., 1983), "NPT II" represents the neomycin phosphotransferase gene of the Tn5 transposon of *E. coli* (Rothstein et al., 1981), "35S pro" represents the 35S promoter isolated from cauliflower mosaic virus (Odell et al., 1985), "BRX" represents the nitrilase gene isolated from *K. ozaenae* (Stalker et al., 1988), "RB" and "LB" represent, respectively, the right-hand and left-hand ends of the sequence of an *Agrobacterium tumefaciens* Ti plasmid.

pRPA-RD-184: Addition of a new, unique restriction site into pRPA-RD-174.

The complementary synthetic oligonucleotides Oligo 7 and Oligo 8 below are hybridized and made double-stranded according to the procedure described for pRPA-MD-P.

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Oligo 7: 5' CCGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC
CCCGGCGCGC CTAGGTGTGT GCTCGAGGGC CCAACCTCAG
TACCTGGTTC AGG 3'

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Oligo 8: 5' CCGGCCTGAA CCAGGTACTG AGGTTGGGCC CTCGAGCACA
CACCTAGGCG CGCCGGGGGCC GCGTTTAAAC TTAATTAAGT
GTGGCCTGAC TGG 3'

5 The hybridized double-stranded
oligonucleotide (96 base pairs) is purified after
separation on agarose gel (3% Nusieve, FMC). The
plasmid pRPA-RD-174 is digested with the restriction
enzyme *XmaI* and the main DNA fragment is purified. The
10 two DNA fragments obtained are then linked together.

A plasmid derived from pRPA-RD-174 is
obtained, comprising other restriction sites between
the bromoxynil-tolerance gene and the selection marker
kanamycin gene.

15 The schematic structure of the plasmid pRPA-
RD-184 is represented in Figure 5, in which the terms
"nos", "NPT II", "NOS pro", "35S pro", "BRX gene", "RB"
and "LB" have the same meanings as in Figure 4.

20 **pRPA-RD-236**: Creation of an *Agrobacterium tumefaciens*
vector containing the gene construct coding for
androctonine directed towards the extracellular matrix.

The plasmid pRPA-RD-233 is digested with the
restriction enzymes *PmeI* and *AscI* and the DNA fragment
25 containing the PR-1a-androctonine gene is purified. The
plasmid pRPA-RD-184 is digested with the same
restriction enzymes. The DNA fragment containing the
PR-1a-androctonine expression cassette is then inserted

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into pRPA-RD-184. An *Agrobacterium tumefaciens* vector containing the sequence coding for the PR-1a-androctonine fusion protein is thus obtained, which leads to the expression of androctonine in the plant's extracellular matrix.

Example 2: Tolerance to herbicides of transformed tobacco plants.

2.1- Transformation

10 The vector pRPA-RD-236 is introduced into the *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1987) carrying the cosmid pTVK291 (Komari et al., 1986). The transformation technique is based on the procedure by Horsh et al. (1985).

15 **2.2- Regeneration**

Regeneration of the tobacco plant PBD6 (obtained from SEITA France) from foliar explants is carried out on Murashige-Skoog (MS) base medium comprising 30 g/l of sucrose and 200 µg/ml of kanamycin. The foliar explants are taken from plants cultivated in a greenhouse or in vitro and regenerated according to the foliar disc technique (Horsh et al., 1985) in three successive steps: the first step comprises induction of the shoots on a medium supplemented with 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 2 weeks. The shoots formed during this step are then grown for 10 days by

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cultivating on MS medium supplemented with 30 g/l of
sucrose but containing no hormone. Next, the shoots
which have grown are taken and cultivated on an MS
rooting medium with half the content of salts, vitamins
5 and sugar and containing no hormone. After about 2
weeks, the rooted shoots are placed in a greenhouse.

2.3- Tolerance to bromoxynil

Twenty transformed plants were regenerated
and placed in a greenhouse for the construction of
10 pRPA-RD-236. These plants were treated in the
greenhouse, at the 5-leaf stage, with aqueous Pardner
suspension corresponding to 0.2 kg of bromoxynil active
material per hectare.

All the plants showing complete tolerance to
15 bromoxynil are then used in various experiments which
show that the expression of androctonine by the
transformed plants makes them resistant to fungal
attack.

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REFERENCES

- Ausubel, F.A. et al., (eds. Greene). Current Protocols
in Molecular Biology. Publ. Wiley & Sons.
- 5 Bevan, M. et al., (1983). Nuc. Acids Res. 11:369-385.
- Carrington and Freed (1990). J. Virol. 64:1590-1597.
- Ehret-Sabatier et al., (1996). The Journal of
Biological Chemistry, 271, 47, 29537-29544.
- Horsch et al., (1985). Science 227:1229-1231.
- 10 Jefferson et al., (1987). EMBO J. 6:3901-3907.
- Komari et al., (1986). J. Bacteriol. 166:88-94.
- Rothstein et al., (1981). Cold Spring Harb. Symp.
Quant. Biol. 45:99-105.
- Stalker et al., (1988). J. Biol. Chem. 263:6310-6314.
- 15 Odell, J.T. et al., (1985). Nature 313:810-812.

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